USE OF SINGLE NUCLEOTIDE POLYMORPHISM ARRAYS TO IDENTIFY A NOVEL REGION OF LOSS ON CHROMOSOME 6Q IN SQUAMOUS CELL CARCINOMAS OF THE ORAL CAVITY

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Abstract: *Background.* A subset of patients with oral cavity squamous cell carcinoma (SCC), often of young age yet lacking a history of carcinogen exposure, has been identified, with no clear etiology for tumor development.

Methods. To identify somatic genetic alterations unique to this patient population, we performed a high throughput single nucleotide polymorphism (SNP) analysis, quantitative PCR of the E6 and E7 regions of human papillomavirus (HPV) 16, sequencing of the IVSF-4+ locus of the FANC-C gene, and microsatellite analysis for 18 nonsmoking patients, age 23 to 57 years (median age, 39 years). We compared these results with oral SCC from 17 patients 47 to 81 (median, 64) years of age with significant tobacco exposure (>40 pack-years) to identify unique genetic alterations for each group.

Results. SNP analysis demonstrated variable rates of allelic imbalance (AI) and no significant difference in terms of AI patterns between the two groups. However, we found an elevated rate of AI in chromosomal arms 6q (47% [17 of 36]) by performing microsatellite analysis of both groups. Only one tumor demonstrated the presence of HPV 16, and none of the tumors demonstrated mutations in the IVSF-4+ region of FANC-C.

Conclusions. Despite variable marker density, SNP array analysis is an emerging technique for genome-wide assessment and is a useful tool for discovery of novel sites of allelic loss in oral SCC, including a novel region of allelic loss on chromosome 6q.
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Squamous cell carcinoma (SCC) of the head and neck affects more than 37,800 people in the United States per year, with more than 20,000 of these cases occurring specifically in the oral cavity. As with other solid tumors, these cancers are thought to arise as a result of a multistep process involving multiple genetic alterations. More than 75% of these cancers are related to carcinogen exposure, usually chronic tobacco and alcohol use. The molecular genetics of head and neck cancer have been

extensively studied; an allelotype for head and neck cancer has been performed, in which a high proportion of loss of heterozygosity/allelic imbalance (LOH/AI) was noted on chromosomal arms 3p, 3q, 9p, 11q, 13q, and 17p.[1]

Recently, however, it has been demonstrated that variations in demographic and phenotypic presentation of head and neck squamous carcinoma (HNSC) may reflect underlying genotypic differences. The subset of nonsmokers among patients with HNSC includes a higher proportion of women whose mean age at diagnosis tends to be at the extremes of age, either very young or very old. Also, the site of the primary lesion in nonsmoking patients with HNSC is preferentially located in the oropharynx and oral cavity, in particular the lateral tongue. At the genetic level, the tumors of nonsmoking patients exhibit overall fewer of the common chromosomal alterations than those of smokers, specifically on chromosomes 3p, 4q, and 11q13; p53 mutations are also less common in the tumors of nonsmokers.[2]

Another phenotypic variation in patients with HNSC involves association with the human papillomavirus (HPV). The role of HPV in HNSC has been an area of interest for decades, and an association between HPV type 16 and oropharyngeal cancer has been demonstrated.[3,4] Among smokers, the prevalence of HPV type 16 is lower than that of nonsmokers with HNSC.[5] Lesions involving HPV type 16 are also more often located in the oropharynx compared with other sites, although recent reports have demonstrated a low prevalence of HPV 16 in oral SCC in general.[6]

Recently, there has been association made between the role of genes involved in the etiology of Fanconi anemia (FANC) and malignancy, specifically HNSC.[7] Alterations in the FANC-C gene have been implicated in hereditary cancer syndromes, with an increased incidence of oral cavity SCC. More specifically, a mutation at the IVSF-4+ locus has been described and implicated in this disorder.

In this study, we examined two specific subsets of patients with HNSC: those with squamous cell carcinoma of the oral cavity with a documented medical history negative for tobacco use compared with another group with typical carcinogen exposure. We performed an initial genome-wide screening with DNA microarray technology specific for single nucleotide polymorphisms (SNPs). Then, regions of loss identified by the SNP microarray screening were further examined by use of established methods for microsatellite analysis. Ancillary studies were performed to further characterize these patients. These include quantitative polymerase chain reaction (PCR) for HPV 16 and sequencing of the FANC-C gene to identify possible mutations at the IVSF-4+ locus. This approach identified a novel region of LOH/AI on chromosome 6q, indicating a region at which a putative tumor suppressor gene may reside.

MATERIALS AND METHODS

Patient Selection. After institutional review board approval, patients were identified from a database of patients with head and neck cancers; they were selected on the basis of age, tumor site, and documented history and physical examination information obtained from their medical records. Patients were divided into two groups: (1) NS: nonsmoking, nondrinking with documented history negative for tobacco use and ethanol intake and (2) S, patients with history of greater than 40 pack-years smoking. Table 1 shows the demographic information for the two groups, as well as the primary site of malignancy. The NS group consisted of nine women and nine men, with a mean age of 37 years, no smoking history, and no consistent alcohol use (one drink per day or more). The S group consisted of seven women and nine men, with a mean age of 63 years and documented smoking history of more than 40 pack-years. With the exception of one patient (patient 14) whose sample was from tumor recurrence, tumor samples were from the initial presentation and surgical resection.

DNA Preparation. Tissue specimens were obtained from archival paraffin-embedded blocks. After examination of 5-μm presample and postsample sections by a board-certified pathologist (WHW), representative tumor and normal, nonmucosal tissue was microdissected from 12 10-μm sections. To remove any residual paraffin, microdissected samples were first incubated in xylene for 6 to 12 hours. After centrifugation and removal of xylenes, samples were incubated in 1% sodium dodecyl sulfate-proteinase K solution for 24 to 48 hours at 48°C. DNA was extracted by use of buffered phenol-chloroform and ethanol, as described previously.[7]

HuSNP Assay

DNA Amplification. Analysis with HuSNP microarrays was performed as described in the Affymetrix GeneChip HuSNP Mapping Assay User Manual

(Affymetrix, Santa Clara, CA). Genomic DNA was amplified in a 24-well multiplex PCR reaction using 5 ng of DNA, 1.25 U of AmpliTaq Gold with supplied buffer (Applied Biosystems, Foster City, CA), 5 m*M* MgCl2, and 0.5 m*M* dNTPs per 12.5 μL reaction. Primers were supplied in the GeneChip HuSNP Reagent Kit (Affymetrix, Santa Clara, CA). PCR conditions were as follows: 95°C for 5 minutes; then 30 cycles of 95°C for 30 seconds, 52°C + 0.2°/cycle for 50 seconds, and 72°C for 30 seconds; then 5 cycles of 95°C for 30 seconds, 58°C for 50 seconds, and 72°C for 30 seconds; then 72°C for 7 minutes. A 1:1000 dilution was performed by adding 1 μL of PCR product to 999 mL of ddH_2O. Then, 2.5 μL of the diluted product was subjected to a second PCR reaction with 4 m*M* $MgCl_2$, 0.4 m*M* dNTPs, and 0.8 μm biotinylated-T3 and biotinylated T7 primers, also provided in the GeneChip HuSNP Reagent Kit. Conditions for the labeling PCR reaction are as follows: 95°C for 8 minutes; then 40 cycles of 95°C for 30 seconds, 55°C for 90 seconds, and 72°C for 30 seconds; then 72°C for 7 minutes; 1.5 μL of product from each pool of the labeling PCR reaction was tested on a 4% agarose gel to confirm the size of approximately 100 bp (BioWhittaker Molecular Applications, Rockland, ME); the remaining products were then pooled and concentrated using a Microcon-10 spin column (Amicon Bioseparations, Bedford, MA).

Table 1. Demographic data for patients and tumor samples studied.

Patient no.	Group	Sex	Age at diagnosis	Tumor site	Pathologic stage
1	NS	M	43	Tongue	T1N0M0
2	NS	F	38	Tongue	T1N0M0
3	NS	F	23	BOT	T2N2M0
4	NS	M	26	BOT	T3N0M0
5	NS	M	29	Tongue	T1N0M0
6	NS	F	25	Tongue	T1N2M0
7	NS	M	32	Tongue	T1N0M0
8	NS	F	40	Tongue	T1N2M0
9	NS	F	48	Tongue	TisN0M0
10	NS	M	57	Tongue	T2N0M0
11	NS	F	40	Tongue	T1N0M0
12	NS	M	34	Mandible	T1N0M0
13	NS	M	28	Tongue	T2N0M0
14	NS	M	43	FOM	Tumor recurrence
15	NS	F	50	Tongue	T1N0M0
16	NS	F	20	Tongue	T1N0M0
17	NS	M	42	Tongue	T1N0M0
18	NS	F	51	Tongue	T1N0M0
19	S	F	70	FOM	T4N2bM0
20	S	F	54	FOM	T2N1M0
21	S	F	62	Tongue	T4N1M0
22	S	M	67	FOM	T1N0M0
23	S	M	66	FOM	T2N0M0
24	S	M	69	FOM	T2N0M0
25	S	F	71	Tongue	T2N2M0
26	S	M	56	Buccal	T4N0M0
27	S	F	65	FOM	T4N0M0
28	S	M	70	FOM	T2N1M0
29	S	F	81	BOT	T2N2M0
30	S	M	47	Tongue	T1N2M0
31	S	M	72	Tongue	T4N2M0
32	S	M	55	FOM	T2N3M0
33	S	M	62	Tongue	T2N2M0
34	S	M	52	FOM	T3N2bM0
35	S	F	59	FOM	T1N0M0

Abbreviations: M, Male; F, Female; BOT, bottom of tongue; FOM, front of mouth.

Hybridization. One hundred five microliters of hybridization mix containing 3 *M* tetramethylammonium chloride (TMAC), 10 m*M* TRIS-HCl pH7.8, 2 n*M* control oligonucleotide B1 (Affymetrix, Santa Clara, CA), 0.01% Tween 20, 5 m*M* EDTA, pH 8.0, and 5× Denhardt's Solution (Sigma, St. Louis, MO) 105 μL was combined with 30 μL of concentrated PCR product. Samples were denatured at 95°C for 5 to 10 minutes, followed by incubation on ice for 2 minutes before loading on HuSNPs. Chips were hybridized at 44°C for 16 hours, rotating at 40 to 50 rpm.

Washing, Staining, and Scanning. After overnight hybridization, each chip was loaded into the Affymetrix fluidics station and subjected to the HuSNP washing and staining protocols. The first wash consisted of two cycles of two mixes per cycle with 6× SSPE at 25°C; the second wash consisted of six cycles of five mixes per cycle with 4× SSPE at 35°C. After the wash protocols, chips were stained with 500 mL staining buffer mix that consisted of 6× SSPE, 1× Denhardt's solution, 0.01% Tween 20, 50 mg/mL streptavidin phycoerythrin (Molecular Probes, Eugene, OR), and 2.5 μg biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA). After the staining protocol, chips were scanned on the GeneArray Scanner (Agilent Technologies, Palo Alto, CA) with the HuSNP scanning protocol.

Data Analysis. Data were analyzed with the Affymetrix GeneChip 4.0 software, and further analysis was performed with data generated by the Microarray Suite Software Analysis Program (Affymetrix, CA). For each SNP locus, a call of AA,

AB, BB, AB_A (AB or A), AB_B (AB or B), or "no signal" was made. The call rate was defined as the proportion of SNPs that were assigned AA, AB, or BB compared with the overall number of SNPs (#). SNPs considered to be informative exhibited both alleles (AB). SNPs with ambiguous calls AB_A or AB_B (AB or A and AB or B, respectively) were placed in the category with no signal. Calls for normal and tumor samples were compared at each SNP. Samples in which both normal and tumor exhibited the AB allele were considered to have retained heterozygosity. Samples in which the normal exhibited both alleles (AB) and the tumor exhibited only one (AA or BB) were considered to have LOH/AI. Samples in which either the normal or tumor was assigned no signal were considered to be noninformative. A call of AA, AB, BB, AB_A, AB_B, or "no signal" at each SNP locus was made after scanning the microarray. The call rate was defined as the proportion of SNPs that were assigned AA, AB, or BB compared with the overall number of SNPs (#). Overall, the call rate for all samples was 62.1%, with no differences between the NS/ND group (61.4%) and the S/D group (62.8%). Of these, approximately 236 of 1494 (15.8%) were informative (236 of 1494 [15.8%] and 236 of 1494 [15.8%] for the NS/ND and S/D groups, respectively).

SNP Mapping. Each SNP was mapped to a chromosomal arm by use of the Human SNP Database (http://www-genome.wi.mit.edu/SNP/human), NCBI Human Genome BLAST page (http://www.ncbi.nlm.nih.gov/BLAST/), Human Genome Browser (http://www.genome.ucsc.edu/), and SNP marker mapping information provided by Affymetrix. For a sample to exhibit informativity on a chromosomal arm, at least one of the SNPs assigned to that arm was read as heterozygous during data analysis. As of July 28, 2002, 1475 of these SNPs have been successfully mapped. SNPs whose location could not be identified or those that were assigned to more than one locus were eliminated from consideration.

Detection of HPV. Reverse transcriptase (RT)-PCR detection of HPV type 16 E6 and E7 DNA was performed on genomic DNA extracted from 14 nonsmoking and nondrinking patients in accordance with the protocol of Ha et al.[6] Specific primers for the E6 and E7 regions of HPV type 16 were used to amplify genomic DNA in a real-time quantitative PCR reaction. Positivity was determined by use of standard curves generated by the use of CaSki cell line genomic DNA, which contains a known number of HPV copies per genome equivalent.

Table 2. Rates of Allelic imbalance by SNP Analysis in HNSC.

	No. patients (%) by group			
Chromosome	SNP−NS	SNP−S	SNP−NS+S	Nawroz et al[1]
1p	5/18 (28)	5/17 (29)	10/35 (29)	7/23 (30)
1q	4/18 (22)	5/17 (29)	9/35 (26)	5/22 (23)
2p	5/18 (28)	4/17 (23)	9/35 (26)	4/21 (19)
2q	5/18 (28)	6/17 (35)	11/35 (31)	3/20 (15)
3p	5/18 (28)	4/17 (23)	9/35 (26)	18/27 (67)
3q	5/18 (28)	0/17 (0)	5/35 (14)	14/28 (50)
4p	2/18 (11)	3/17 (18)	5/35 (14)	8/21 (38)
4q	4/18 (22)	4/17 (23)	8/35 (23)	9/24 (37)
5p	4/9 (44)	1/12 (8)	5/21 (24)	5/26 (19)
5q	6/18 (33)	5/17 (29)	11/35 (31)	5/20 (25)
6p	5/18 (28)	6/17 (35)	11/35 (31)	9/24 (38)
6q	8/18 (44)	1/17 (6)	9/35 (26)	6/26 (23)
7p	2/17 (12)	2/14 (14)	4/31 (13)	6/26 (23)
7q	5/18 (28)	2/17 (12)	7/35 (20)	7/24 (29)
8p	6/18 (33)	6/17 (35)	12/35 (34)	8/20 (40)
8q	4/18 (22)	4/17 (23)	8/35 (23)	10/26 (38)
9p	4/15 (27)	4/16 (25)	8/31 (26)	21/29 (72)
9q	8/18 (44)	9/17 (53)	8/35 (23)	3/23 (13)
10p	1/10 (10)	1/12 (8)	2/22 (9)	5/22 (23)
10q	5/18 (28)	6/17 (35)	11/35 (31)	5/24 (21)
11p	6/18 (33)	5/17 (29)	11/35 (31)	4/24 (17)
11q	7/18 (39)	5/17 (29)	12/35 (34)	14/23 (61)
12p	2/18 (11)	1/17 (6)	3/35 (9)	5/28 (18)
12q	3/18 (17)	3/17 (18)	6/35 (17)	6/24 (25)
13p	0/3 (0)	0/6 (0)	0/9 (0)	
13q	7/18 (39)	6/16 (37)	13/34 (38)	12/22 (54)
14p	1/8 (12)	1/9 (11)	2/17 (12)	
14q	2/17 (12)	1/17 (6)	3/34 (9)	9/23 (39)
15p	1/8 (12)	1/5 (20)	2/13 (15)	
15q	6/18 (33)	4/17 (23)	10/35 (29)	1/21 (5)
16p	2/14 (14)	1/16 (6)	3/30 (10)	2/20 (10)
16q	4/17 (23)	5/17 (29)	9/34 (26)	5/25 (20)
17p	7/16 (44)	6/14 (43)	13/30 (43)	12/23 (52)
17q	8/18 (44)	2/17 (12)	10/35 (29)	8/26 (31)
18p	0/15 (0)	2/13 (15)	2/28 (7)	6/22 (27)
18q	3/18 (17)	2/17 (12)	5/35 (14)	6/26 (23)
19p	2/18 (11)	1/17 (6)	3/35 (9)	6/19 (32)
19q	4/17 (23)	1/17 (6)	5/34 (15)	10/25 (40)
20p	1/17 (6)	1/16 (6)	2/33 (6)	6/20 (30)
20q	0/12 (0)	1/14 (7)	1/26 (4)	2/21 (9)
21p	1/1 (100)	0/1 (0)	1/2 (50)	
21q	3/18 (17)	2/17 (12)	5/35 (14)	5/19 (26)
22p	0/8 (0)	0/1 (0)	0/9 (0)	
22q	1/17 (6)	0/17 (0)	1/34 (3)	7/24 (29)
Xp	0/7 (0)	0/5 (0)	0/12 (0)	
Xq	0/4 (0)	0/4 (0)	0/8 (0)	

FANC-C Gene Sequencing. PCR primers were designed for exon 4 of the Fanconi anemia C gene and included the site of the IVSF-4 mutation. Forward and reverse primers for DNA amplification were as follows: 5′-GTAGGCATTGTACATAAAAG-3′ and 5′-TGGCACATTCAGCATTAAAC-3′. The region of interest was amplified with 250 ng each of forward and reverse primers, 1.5 units TAQ polymerase, 2.5 μL of PCR buffer, and 1.5 μL DMSO in a 25-μL PCR reaction at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, and 72°C for 5 minutes. PCR products were electrophoresed on a 1% agarose gel and purified by use of a commercially available kit (Qiagen, Valencia, CA). Manual sequencing was performed with the Amplicycle Sequencing kit (Perkin Elmer, Wellesley, MA) with sequencing primer as follows: 5′-TCAGCACTCAGATTTGATAAAG-3′.

Microsatellite Analysis. Microsatellite markers THRB, IGF2R, D3S1284, D6S87, D9S59, D9S156, D9S199, D9S200, and D9S310 were selected for comparison with HuSNP data; 50 ng of forward primer was first radiolabeled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), 1× PNK buffer and 20 μCi γ-^{32}P dATP at 37°C for 30 minutes. In each 12.5 μL reaction, 50 ng of genomic DNA was amplified with 25 m*M* dNTPs, 0.5 U Taq polymerase (Invitrogen Corporation, Carlsbad, CA), and 1.0 ng γ-^{32}P-labeled primer. The PCR buffer included 16.6 m*M* ammonium sulfate, 67 m*M* TRIS (pH 8.8), 6.7 m*M* magnesium chloride, 10 m*M* β-mercaptoethanol, and 0.1% dimethyl sulfoxide. PCR cycling was as follows: 95°C for 2 minutes and then 30 cycles of 95°C for 30 seconds, 57°C for 1 minute, 70°C for 1 minute; and 70°C for 7 minutes. One fifth of the PCR product was then electrophoresed on a 6% polyacrylamide gel at 120 watts for 3.5 hours and exposed to photographic film overnight. Allelic imbalance or loss of heterozygosity was defined as decreased intensity of one allele of the tumor DNA less than 50% that of the corresponding allele of the normal DNA by inspection (by BCT), confirmed by a second observer (JC), and confirmed again on repeat amplification and gel electrophoresis.

RESULTS

We searched for somatic genetic alterations in SCC from 18 nonsmoking (NS) and 17 smoking (S) patients. The age range of the NS group was 23 to 57 years (median, 39 years) compared with 47 to 81 years (median, 64 years) for the S group. All patients from the NS group were never smokers, and all patients from the S group had at least 40 pack-years of cigarette exposure.

We tested 36 tumors by use of microsatellite analysis, 19 from the NS group and 17 from the S group. Table 2 shows the rates of informativity and AI for each tumor group and chromosomal arm. These are compared with a distinct group of historical controls published in previous studies.[1]

Allelic imbalance of chromosome 6q was 44% by SNP array analysis for NS, far exceeding the 6% rate reported by SNP analysis for the S group. However, the of AI demonstrated on SNP analysis

Table 3. Allelic imbalance by Microsatellite analysis.

	No. patients (%) by group				
Chromosomal arm/marker	Nawroz et al[1]	SNP–NS	SNP–S	MS–NS	MS–S
3p	18/27 (67)	5/18 (28)	4/16 (25)	8/17 (47)	10/14 (71)
THRB	6/23 (26)			6/11 (55)	5/9 (56)
D3S1284	12/19 (63)			2/12 (17)	7/11 (64)
6q	6/26 (23)	8/18 (44)	1/16 (6)	5/16 (31)	2/14 (14)
IGF2R				3/15 (20)	1/7 (14)
D6S87				3/12 (25)	2/13 (15)
9p	21/29 (72)	4/15 (27)	3/15 (20)	13/19 (68)	10/17 (59)
D9S156	13/18 (72)			6/18 (33)	4/10 (40)
D9S199	15/21 (71)			8/16 (50)	5/17 (29)
D9S200	13/25 (52)			8/19 (42)	8/12 (67)
9q	3/23 (13)	8/18 (44)	9/16 (56)	5/17 (29)	7/16 (44)
D9S310				4/13 (31)	4/11 (36)
D9S59				2/13 (15)	5/10 (50)

were discrepant from those reported for many chromosomal arms. For example, the rates reported for chromosome 9p and 3p are 72% and 67% by microsatellite analysis, respectively, and 26% percent each by SNP analysis.

To reconcile the discrepancy between microsatellite-based estimation of AI and SNP array-based data, we performed microsatellite analysis with identical markers to those used in prior reports of HNSC allelotypes for selected chromosomal arms (Table 3). Microsatellite analysis demonstrated AI rates of 19% (7 of 36) and 33% (12 of 36) for 6q and 9q, respectively, for all 36 tumors (NS and S groups combined). In addition, standard microsatellite analysis revealed a high frequency of AI on chromosomal arms 3p and 9p on the identical tumor set (56% and 64%, respectively).

Finally, additional mapping by use of microsatellite markers was performed to delineate an area of minimal loss on chromosome 6q (Figure 1). Complex patterns of AI were noted; however, areas of apparent retention flanked by AI indicating the possibility of homozygous deletions were concentrated in the central part of the chromosomal arm. One tumor (626) demonstrated an isolated AI at 6q23 at marker D6S270, a second tumor (1850) demonstrated an isolated AI at D6S239, and another tumor (1578) showed a single marker with AI at 6q25 at marker D6S220. These complex patterns of AI indicate that focal targeting of small regions occur in this chromosomal arm, but that other alterations, including translocations, may hinder efforts at fine mapping without analysis of a larger cohort of tumors.

The high rate of AI at 9q prompted us to analyze the FANC C gene, responsible for an inherited cancer syndrome with an elevated rate of oral SCC. Specific mutations have been described for the FANC C gene on chromosome 9q, including the IVSF-4+ locus mutation.[7–9] However, none of the 36 tumor samples exhibited mutations in the IVSF-4+ locus associated with mutations in the FANC-C gene. In addition, none of 14 NS and one of 17 S samples tested positive for the presence of HPV 16 E6 and E7 proteins. Thus, HPV 16 infection and FANC C IVSF-4+ mutation do not seem to be significant etiologic factors in the development of these tumors studied.

DISCUSSION

SNP Analysis. This study describes the use of DNA-based SNP microarrays to screen for LOH/AI in a select population of patients with SCC of the oral cavity. After an initial genome-wide screening, chromosomal regions of interest were further explored by use of traditional microsatellite analysis and gene sequencing. One advantage of the use of DNA microarray technology is the ability to screen the entire genome with small amounts of genomic DNA. Ideally, SNP microarrays would facilitate a rapid and detailed analysis of AI in human tumors. This would facilitate discovery of novel areas of AI that may harbor potential tumor suppressor genes or amplified proto-oncogenes. We were able to identify chromosomal arm 6q as an area of increased AI with SNP arrays and confirmed this finding with conventional microsatellite analysis. However, chromosomal arms with previously known, higher rates of AI were underestimated using SNP analysis. Microsatellite analysis with markers in selected regions of frequent AI indicated that high rates of AI did exist for these chromosomal arms in this tumor set. This indicates that SNP arrays, although a useful tool for discovery of novel areas of AI, may underestimate loss in a chromosomal arm if the informative SNP markers are not close to the minimal regions of high AI or if the density of coverage of an SNP marker panel is not sufficiently dense to provide complete evaluation of a chromosomal arm. One possible reason for this phenomenon may be the nonrandom distribution of SNPs across the various chromosomes. For example, we mapped 114 SNPs to chromosome 1, whereas there were only 23 SNPs mapped to chromosome 21. In some instances, this is probably due to a no-signal genotype call either in tumor or in normal DNA or in both. This problem may be addressed by increasing the density of SNPs for the specific loci and by developing a more sensitive algorithm for the generation of calls. In addition, the call rate of 62% is lower than that reported in other studies; it is possible that this reflects a slightly poorer DNA quality resulting from extraction from paraffin-derived tissue, despite the fact that electrophoresis demonstrated that SNP assay amplification products were of the appropriate approximate 100-bp size.[10,11] Finally, precise physical localization of SNP loci is still incomplete. In many cases, the SNPs have yet to be mapped; in others, there were multiple possible loci for the SNPs, which was a basis for excluding that SNP from consideration in our study. However, the effect of incorrect localization for a minority of SNPs did not seem to be great, and

it is expected that this will improve in the future as SNPs become more precisely mapped. Potential future applications to address these issues would also include high-density SNP arrays dedicated to single chromosomes that can be used to facilitate fine mapping after initial discovery of areas of high AI in single chromosomal arms. Finally, low rates of AI detection in selected chromosomal arms by SNP array may be due to small sample size ($n = 36$). However, validation of high rates of AI in these same samples was easily demonstrated by conventional microsatellite analysis, indicating that this was a limitation of the assay.

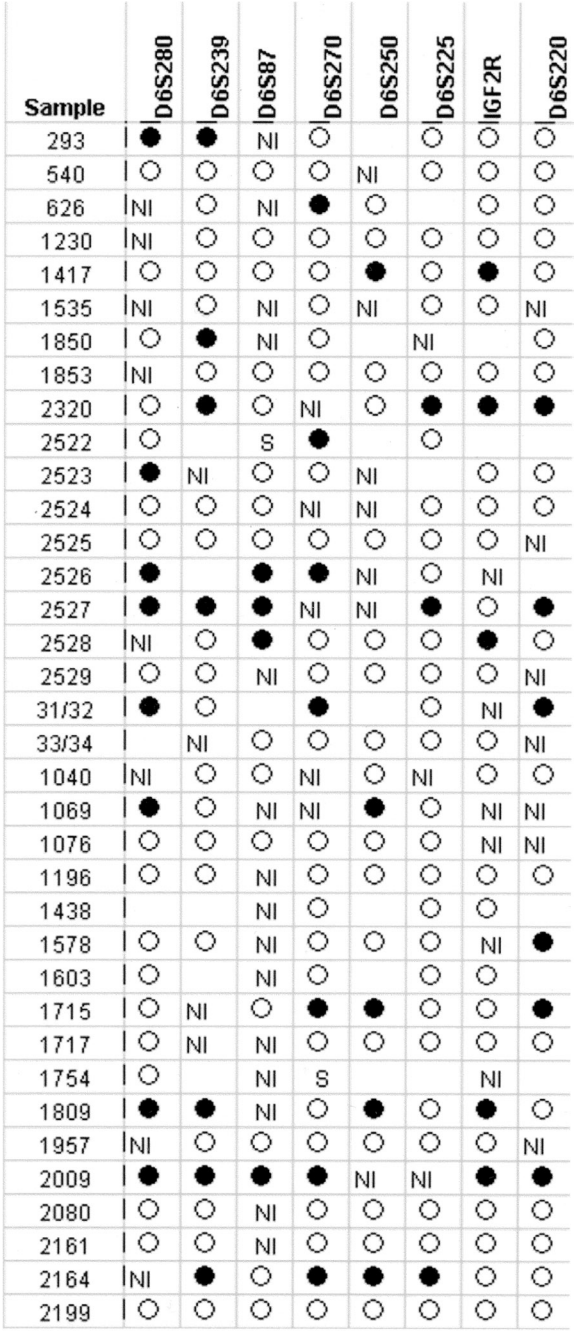

FIGURE 1. Microsatellite analysis of chromosome 6q. Upper legends denote microsatellite markers; tumor sample identification number identifies each row. Filled circles = allelic imbalance; open circles = retention of heterozygosity; NI = noninformative; empty boxes = not done.

Because this study was conducted as an initial exploration of the use of SNP technology in screening HNSC tumors for AI, our sample sizes were small, and statistical analysis was not performed. In part, we were limited by the relatively small number of patients who were initially seen with HNSC at a young age and with no history of tobacco use. Certainly, further studies are needed to clarify the molecular genetic differences between tumors from these patients and those with significant tobacco and alcohol use.

None of the samples from either group exhibited mutations at the FANC-C IVSF-4+ locus. However, it is possible that other defects in downstream processing or expression could contribute to the development of HNSC in a population without other classic risk factors.

None of the samples from the nonsmoker/nondrinker group tested positive for HPV 16, essentially eliminating this as a cause of these tumors in this cohort.

This study does confirm that 6q is a chromosomal arm with significant AI, consistent with prior reports based primarily on cytogenetic data.[12–14] Although a single tumor with AI isolated to 6q26 is described in this series, the pattern found in other tumors is more complex, and additional fine mapping would be required to confirm this as a minimal area of allelic imbalance. Previous work has identified complex patterns of loss in HNSC on chromosome 3p, and it is possible that our findings are a reflection of similar processes producing these complex patterns.[15,16] Finally, for this analysis, SNP arrays in their current form seem to be a useful tool for discovery of novel areas of AI but seem to have limitations in terms of fine mapping and determination of definitive rates of AI. Further refinements in this technique will likely provide significant and rapid improvements to the characterization of AI in solid tumors using SNP microarrays.

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